Sanitizing food contact surfaces by the use of essential oils

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ABSTRACT

Chemical sanitizers continue to be widely used by the food industry to disinfect food contact surfaces. However, as some chemical disinfectants have been reported to produce unhealthy by-products, alternative and natural compounds need to be investigated. To this end, nine essential oils (EOs) were screened to develop a natural sanitizing solution (SAN) for disinfecting food contact surfaces. Once extracted, their antimicrobial activity and chemical composition were determined. An exploratory multivariate approach was used to investigate the relationships between the chemical and microbiological data sets. Among the tested EOs, *Thymbra capitata* EO, containing up to 93.31% oxygenated monoterpenes (mainly carvacrol), showed the strongest antimicrobial activity and thus was assayed as a potential SAN for food contact surfaces. To this end, a SAN consisting of 1% *T. capitata* EO was first validated according to the AOAC standard, which showed about an 8 log reduction for *Escherichia coli* and *Salmonella enterica* after 30 and 60 seconds of contact time, respectively. Then, the SAN was evaluated at various concentrations, cleanliness conditions, and contact times on stainless steel, glass, and polypropylene surfaces for sanitizing purposes. The results showed that the SAN containing 2.5% of *T. capitata* EO applied for 10 min, reduced the levels of *E. coli* by more than 3 log and *S. enterica* by 1 log under clean working conditions on the three tested surfaces. These findings indicate that EOs can be used as natural disinfectants to decontaminate food contact surfaces, thus lowering the risk of the indirect transfer of bacterial pathogens to food or persons.

Keywords: Essential oils; Natural sanitizers; Foodborne pathogens; Food contact surfaces; Food safety.

1. Introduction

Microbial safety of food products is a key concern of consumers, the food industry, and regulatory bodies. Thus, different guidelines have been proposed to limit and control the occurrence of pathogens in food products (Codex alimentarius, 2007), and they agree that these risks can be reduced through safe food preparation, consumption, and storage practices by increasing hygienic measures along the entire food chain. On top of that, diarrheal diseases caused by bacteria are one of the most common illnesses resulting from the consumption of contaminated food (World Health Organization, 2014).

In the European Union, *Salmonella* and Shiga toxin-producing *Escherichia coli* have been identified as the first and seventh most common causes of foodborne illness outbreaks, respectively (EFSA, 2016). Moreover, *Salmonella* and *E. coli* are considered safety/hygiene indicators because their presence in food and water is due to fecal contamination and\or inadequate hygiene practices (Ceuppens et al., 2015).

The role that contaminated surfaces play in spreading pathogenic bacteria such as *Salmonella* and *E. coli* to foods is already well established in food processing, catering, and domestic environments such as chopping boards, knives, processing machines, tanks, and vats that can act as reservoirs and/or vehicles of pathogens. Food contact surfaces and equipment are commonly made by different materials such as stainless steel, and polypropylene glass that can divergently play in harboring pathogens (Chia, Goulter, McMeekin, Dykes, & Fegan, 2009; Duffy, O'Callaghan, McAuley, Fegan, & Craven, 2009).

In the food industry, to reduce the spread of bacteria through contaminated surfaces, chemicals are routinely used to sanitize and disinfect food contact surfaces (Phillips, 2016; Simões, Simões, & Vieira, 2010). However, some of these chemicals (e.g. chlorine compounds, peroxide and peroxyacid mixtures, carboxylic acids, quaternary ammonium compounds, acid anionic, and iodine compounds) may generate the formation of by-products (e.g. trihalomethanes, haloacetic acids, and other potentially carcinogenic compounds), or contribute to the development of antibiotic resistance

in bacteria (e.g. triclosan) (Coroneo et al., 2017; Davidson & Harrison, 2002; Doyle, 2006; Halden, 2014; Marques et al., 2007; Xue et al., 2017). Alternative antimicrobial compounds would, therefore, be beneficial, especially for the development of natural sanitizers. In recent years, because of increased consumer awareness and concern regarding synthetic chemical additives or sanitizers, foods and food-contact surfaces treated with EOs or their main active compounds have become very popular since they are safer for humans and environmentally-friendly (S. Burt, 2004). Moreover, many of them show antimicrobial, antifungal, and virucidal properties, and thus represent potential 'natural' alternatives to chemical preservatives in the food industry (S. Burt, 2004; da Cruz Cabral, Fernández Pinto, & Patriarca, 2013; Sánchez & Aznar, 2015).

The selection and standardization of EOs is a critical task because many factors (e.g. plant material, ecological conditions, and extraction method) affect their chemical composition and, consequently, their biological and antimicrobial properties (S. Burt, 2004; Settanni et al., 2014).

Some EOs such as *Citrus* spp. (Fisher & Phillips, 2008), cinnamon (Van Haute, Raes, Devlieghere, & Sampers, 2017), oregano, and thyme (Yemiş & Candoğan, 2017) have been used as natural antimicrobials in food application, while uncommon, plant-derived EOs have received limited attention. So far, some well characterized EOs or their main active compounds have been directly applied as flavoring agents in food, used in washing solutions for vegetables, or incorporated in packaging materials to control foodborne pathogens (Irkin & Esmer, 2015). Furthermore, the application of well-characterized EOs to sanitize food contact surfaces has also been investigated (Giaouris et al., 2014; Rhoades et al., 2013; Valeriano et al., 2012)

Thus, this study aims to (i) collect, extract, and chemically characterize EOs from little-known plants; (ii) screen their antimicrobial activity against the common foodborne pathogens *S. enterica* and *E. coli*; and (iii) develop a natural EO-based sanitizer and evaluate its antibacterial activity on stainless steel, glass, and polypropylene surfaces.

2. Materials and methods

94 *2.1. Plant material and extraction of EOs and aqueous extracts*

95 Aerial parts (leaves and/or sprigs) from *Eriocephalus africanus* L. (EO1), *Artemisia absinthium* L.
96 (EO2), *Santolina chamaecyparissus* L. (EO3), *Mentha longifolia* (L) L. (EO4), *Thymbra capitata*
97 (L.) Cav. (EO6), *Citrus limon* (L.) Osbeck (EO7), *Citrus reticulata Blanco* (EO8) and *Eucalyptus*
98 *camaldulensis Dehnh* (EO9) were collected in different areas of Spain to obtain their EOs (Table
99 1S). *Pelargonium odoratissimum* (L.) L'Hér. EO (EO5) was purchased from Titolchimica (Italy).
100 After collection, fresh plant material was immediately subjected to hydro-distillation for 3 h using a
101 Clevenger-type apparatus, collecting the oil in hexane. In particular, about 500 g of plant material
102 was weighted and transferred to 4 l Clevenger flasks with 2 l of distilled water. The steam and oil
103 vapour were condensed and the oil was separated from the water using Florentine flasks. EOs were
104 dried over anhydrous sodium sulfate to remove residual water traces and finally the extraction yield
105 calculated (Table 1S). EOs were stored at 4°C in air-tight sealed glass vials covered with aluminum
106 foil until use.

107 *2.2. Chemical characterization of EOs*

108 The quantification of the samples was performed by gas chromatography (GC) using a Clarus
109 500GC Perkin–Elmer apparatus equipped with a flame ionization detector (FID), and capillary
110 column ZB-5 (30 m × 0.25 mm i.d. × 0.25 μm film thickness). The injection volume was 1 μl. The
111 GC oven temperature was set at 60ºC for 5 min, with 3ºC increases per min to 180ºC, then 20ºC
112 increases per min to 280ºC which was maintained for 10 min. Helium was the carrier gas (1.2
113 ml/min). Injector and detector temperatures were set at 250°C. The percentage composition of the
114 EO was computed from GC peak areas without correction factors by means of the software Total
115 Chrom 6.2 (Perkin-Elmer Inc., Wellesley, PA, USA).
116 For the identification of the compounds, gas chromatography coupled to mass spectrometry (GC-
117 MS) was used (Adams, 2007) using a Clarus 500 GC-MS from Perkin-Elmer Inc., equipped with
118 the same column, carrier and operating conditions as described above for GC analysis. Ionization
119 source temperature was set at 200°C and 70 eV electron impact mode was employed. MS spectra

were obtained by means of total ion scan (TIC) mode (mass range m/z 45-500 uma). The total ion chromatograms and mass spectra were processed with the Turbomass 5.4 software (Perkin-Elmer Inc.). Retention indexes were determined by injection of C8–C25 n-alkanes standard (Supelco) under the same conditions. The EO components were identified by comparison of their mass spectra with those of computer library NIST MS Search 2.0 and available data in the literature. Identification of the following compounds was confirmed by comparison of their experimental RI with those of authentic reference standards (Sigma-Aldrich): α-pinene, β-pinene, camphene, myrcene, limonene, camphor, terpinolene, β-thujone, borneol, terpinen-4-ol, bornyl acetate, geraniol and linalool.

2.3. Screening for antimicrobial activity and minimum inhibitory concentration determination

The reference strains *E. coli* O157:H7 CECT 5947 (non-toxigenic) and *S. enterica subsp. enterica* CECT 4138 supplied by the Spanish Type Culture Collection (CECT) were used to test the antibacterial activity of nine EOs. Firstly, paper disc diffusion assay (PDDA) was used as rapid screening method (Balouiri, Sadiki, & Ibnsouda, 2016; Settanni et al., 2014). Briefly, bacterial cells were grown overnight at 37°C on tryptic soy broth (TSB), the concentration adjusted to 7 log CFU/ml and seed on tryptic soy agar (TSA) using a cotton swab. Once dried, sterile paper discs (Sigma-Aldrich) were placed on the plate surface. Each disk was soaked with 10 µl of each undiluted EO. Sterile water and streptomycin (10%, w/v) were used as negative and positive control, respectively. Each test was performed in duplicate and the experiments were repeated twice. Additionally, the minimum inhibitory concentration (MIC) was determined. For that, bacterial cultures of ca. 6 log CFU/ml were exposed to increasing EO concentrations (0, 0.025, 0.05, 0.1, 0.5 and 1%) and incubated overnight at 37°C. Growth inhibition was evaluated after 4 and 24h of incubation by plate count on TSA.

2.4. Evaluation of the EO-based sanitizer following AOAC 960.09 and EN 13697:2015 standards

Based on preliminary antimicrobial assays (PDDA and MIC), a sanitizer solution (SAN) was prepared using EO6 and ethanol mixed in a ratio 1:1. SAN was freshly prepared before each assay.

Initially, the SAN was evaluated following the AOAC 960.09 standard method "Germicidal and detergent sanitizing action of disinfectants". Briefly, 9.90 ml solution of 2 % SAN prepared in synthetic hard water of 400 ppm $CaCO_3$ (AOAC 960.09) was inoculated with 0.1 ml of bacterial inoculum, resulting in a final concentration of ca. 8 log CFU/ml, and incubated for 30 and 60 seconds at room temperature (RT). Then, serial dilutions were performed using peptone water (PW) as neutralizer (previously validated according to the method) and colony forming units (CFU) enumerated on TSA after 24 h at 37ºC.

2.5. Surface disinfection tests

Surface disinfection tests were performed using the EN 13697:2015 standard "Chemical disinfectants and antiseptics. Quantitative non-porous surface test for the evaluation of bactericidal and/or fungicidal activity of chemical disinfectants used in food, industrial, domestic and institutional areas. Test method and requirements without mechanical action". The bactericidal activity of SAN was evaluated on stainless steel, glass and polypropylene discs. Discs (2 x 2 cm) were sterilized with 70% (V/V) of isopropanol for 15 min before each assay. Briefly, *E. coli* and *S. enterica* suspensions were diluted (ratio 1:1) with 0.3 and 3 g/l bovine serum albumin (BSA) to mimic clean and dirty working conditions (as in EN 13697:2015). Then 50 µl of resulting inocula (ca. 6 log CFU/ml) were spotted into sterile discs and dried at RT for 15 min. Afterward, 100 µl of 0.5, 1 and 5% SAN prepared on hard water as diluent according to EN 13697:2015, were spotted on the inoculated discs, followed by incubation at RT for 1, 5 and 10 min. Then, the effect of the SAN was stopped by transferring the discs into a flask with 10 ml of peptone water as neutralizer and 5 g of glass beads. After 1 min in a shaker at 240 rpm (VWR, The Netherlands), bacterial cells were enumerated as described above. Positive controls were performed using discs treated with hard water contained the same ethanol concentration as applied for SAN.

2.6. *Statistical and explorative multivariate analyses*

Data obtained from chemical characterization and antimicrobial activities of EOs were analyzed using an explorative multivariate analysis, including a hierarchical cluster analysis (HCA) and a

principal component analysis (PCA). Firstly, HCA was carried out for grouping EOs samples measured by Euclidean distances; whereas cluster aggregation was based on the single linkage method (Todeschini, 1998). The input matrix used for HCA consisted of chemical compounds and MIC for both *E. coli* and *S. enterica.* The PCA explored the input matrix based on the 9 EOs introduced as cases and the normalized average data of 178 chemical compounds grouped according to their chemical classes and MIC for both *E. coli* and *S. enterica* considered as explanatory variables, preliminary evaluated by using the Barlett's sphericity test (Alfonzo et al., 2017; Bautista Gallego et al., 2011). Eigenvalues were calculated and score and loading plots including both EOs samples and GC-MS constituents were generated (Torregiani et al., 2017). The analysis of variance (ANOVA), followed by a pairwise comparison with the post-hoc Tukey's test, was applied to identify significant differences for SAN efficacies (Figures 3 and 4) with a statistical significance attributed to p values <0.05. All statistical data processing and graphic constructions were performed using STATISTICA software version 7 (StatSoft Inc., Tulsa, OK, USA).

3. Results and discussion

3.1. *Extraction and GC/GC-MS characterization of EOs*

The EOs' extraction yields are reported in Table 1S and ranged between 0.22 for EO2 and 3.00% (v/w) for EO6. Similar extraction yields have already been reported for *E. africanus* (0.43% v/w), *E. camaldulensis* (0.71% v/w) and *T. capitata* (2.1-5.6% v/w) (Bounatirou et al., 2007; Verdeguer, Blázquez, & Boira, 2009). The main chemical compounds constituting more than 10% of the total composition determined by GC/GC-MS for each of the nine EOs are reported in Table 1 whereas the complete composition is reported in Table 2S. A high percentage of compounds were identified for all EOs (92.69 - 99.20%), and they are grouped into different chemical classes as monoterpene hydrocarbons (MH, ranging from 2.05 to 64.47%), oxygenated monoterpenes (OM, from 28.82 to 93.3%), sesquiterpene hydrocarbons (SH, from 0 to 5.05%), oxygenated sesquiterpenes (OS, from 0 to 21.82%) and esters (EST, from 0 to 0.83%).

198 EO1 was mainly characterized by artemisia ketone (57.54%) among MH and intermedeol (10.54%)
199 among OS. For EO2, the OMs epoxy-ocimene <(E)-> (34.01%) and cis-chrysanthenyl acetate
200 (28.35%) were the most abundant among a total of 28 compounds. The OM camphor (31.43%), 1,8-
201 cineole (11.74%), terpinen-4-ol (8.64%), and the OS β-copaen-4-α-ol (10.11%) were the main
202 compounds in EO3. EO4 was mainly characterized by the OM α-terpineol acetate (32.59%),
203 pulegone (14.15%), carvone acetate (10.29%), and isomenthone (9.16%). Citronellol (20.40%), α-
204 terpineol (12.60%) and geraniol (12.30%) were the main compounds of the EO5. EO6 showed 23
205 different compounds (99.31%), with a slight amount of MH (3.51%) and a high percentage of OM
206 (93.06%). Among OM, it is worth noting that carvacrol contributed to a significant percentage of
207 the EO composition (91.56%), while only 0.03% of thymol was detected. This high carvacrol level
208 distinguishes this species from others of the *Thymus* genre (e.g. *Thymus vulgaris)*, which are
209 characterized by high levels of thymol, another OM showing antimicrobial activity (S. Burt, 2004).
210 EO7 showed limonene (30.14%) and β-pinene (17.28%), both MHs, together with geranial
211 (11.91%), an OM, as its main compounds. EO8 was characterized by sabinene (34.41%) and
212 linalool (21.27%). EO9 exhibited a total of 40 compounds; p-cymene (28.34%), cryptone (14.12%),
213 and spathulenol (17.99%) were the most abundant.
214 Comparing the EOs' chemical compositions, the types of compounds and their concentrations
215 showed wide variability due to the botanical diversity of the plant material used for EO extraction.
216 Thus, plant material deeply influences the final EO constituents, their relative concentrations (S.
217 Burt, 2004; Chang, Chen, & Chang, 2001), and, finally, the EO antibacterial activities.
218
219 3.2. *Antimicrobial activity of EOs*
220 The antimicrobial activity of the nine EOs against *E. coli* and *S. enterica* is shown in Table 2. Both
221 PDDA, and MIC determinations identified EO6 as the most effective; it had the widest inhibitory
222 haloes (2.75 and 2.47 cm for *E. coli* and *S. enterica*, respectively) and inhibited the growth of both
223 tested strains at the lowest concentration (MIC of 0.05% v/v). Considering its chemical

224 composition, it could be inferred that carvacrol (comprising 91.56% of the 99.31% identified
225 compounds) was directly responsible for the antimicrobial effect. This finding is not surprising
226 since the antimicrobial activity of carvacrol has already been reported against several foodborne
227 pathogens (Friedman, 2014; Nostro & Papalia, 2012), and resistant isolates (Memar, Raei,
228 Alizadeh, Aghdam, & Kafil, 2017). In addition, similar MIC values (0.025-0.03%) have been
229 reported for pure carvacrol against *S. Typhimurium* (Kamlesh et al., 2013) and *S. enterica* (Engel,
230 Heckler, Tondo, Daroit, & da Silva Malheiros, 2017).
231 In line with these results, the poor antibacterial activity of *E. africanus* (EO1), *A. absinthium* (EO2),
232 and *M. longifolia* (EO4) have already been reported (Anwar, Alkharfy, Najeeb-ur-Rehman, Adam,
233 & Gilani, 2017; Mkaddem et al., 2009; Riahi et al., 2015; Salie, Eagles, & Leng, 1996). EO3,
234 extracted from *S. chamaecyparissus*, showed an MIC of 0.5%, a higher value with respect to the
235 0.0001% v/w reported for *E. coli* by Bel Hadj Salah-Fatnassi et al. (2017). In contrast, EO5,
236 extracted from *P. Odoratissimum,* showed MIC values of 1% for both strains, indicating only
237 moderate activity, while poor antimicrobial activity has been previously reported (Andrade,
238 Cardoso, Batista, Freire, & Nelson, 2011; Lis-Balchin & Roth, 2000).
239 Compared to previous research, poor antibacterial activity (MIC≥0.5% v/v) was found for *Citrus*
240 EOs (EO7 and EO8) (Fisher & Phillips, 2008; Randazzo, Jiménez-Belenguer, et al., 2016; Settanni
241 et al., 2014). These discrepancies can be explained by several factors, such as intrinsic factors of the
242 plants (e.g. genotype and, part of the plant harvested, such as leaves vs peel), harvest time,
243 geographical and ecological conditions, extraction method, and the method for antimicrobial
244 determination, including the types of bacterial strains tested (S. Burt, 2004; Randazzo, Jiménez-
245 Belenguer, et al., 2016). In addition, the structural characteristics of the EOs' active compounds (i.e.
246 aliphatic ring, hydroxyl group) may change depending on the extraction procedure applied and/or
247 storage time, resulting in a different level of antimicrobial activity, such as that reported for
248 carvacrol (Veldhuizen, Tjeerdsma-Van Bokhoven, Zweijtzer, Burt, & Haagsman, 2006).

In case of EO9, a MIC of 0.5% v/v was recorded against *E. coli* according to Nasir, Tafess, and Abate (2015), while Sliti et al. (2015) reported higher values (1.5% v/v for *E. coli* and 1.0% for *S. enteritidis*).

3.3. Explorative multivariate analysis of chemical composition and antibacterial activities

Since HCA gathers cases according to their overall similarity and PCA plots cases and variables together to provide information on their correlation, the two methods are complementary in their ability to present and discuss chemical and microbiological results (Alfonzo et al., 2017; Bendiabdellah et al., 2014; Randazzo, Guarcello, et al., 2016).

HCA mainly classified the EOs into two mega-clusters at around 95% of their mutual dissimilarity (Fig. 1); EO6 was clustered separately from the remaining EOs. In this last group, the EOs shared 66% of dissimilarity with EO1 and only 54% among themselves. In general, the high linkage distance among the cases (>46%) reflects the high complexity of the EOs's chemical composition and antimicrobial activity, which were used as variables for the HCA analysis.

Regarding PCA, EO1 and EO2 were not included in the analysis due to their negligible antimicrobial activity (lowest PDDA values). Four Factors displayed eigenvalues higher than 1, explaining 95.32% of the total variance (Table 3S). In particular, the scatterplots represent the relationship between the three main Factors and EOs (score plot, Fig. 2A), and, between the three main Factors and variables (loading plot, Fig. 2B), accounting for 82.59% of the total variance. Factor 1 represents 33.69% of the total variance and it is positively correlated with OM and negatively correlated with MH, OS and MIC (Fig. 2B and Table 4S). Factor 3 (22.64%) is positively correlated with OM, OS, and MIC variables for both *E. coli* and *S. enterica*; it is the Factor most correlated to the EOs' antimicrobial traits. Similarly, MH, EST, and OTH correlated negatively with Factor 3. Interestingly, EO6 showed the highest correlation value with Factor 3 (associated with antimicrobial traits, Tab. 5S).

In summary, the discrimination of EOs based on the scatterplots highlighted differences among the samples that resulted in widely spaced points (Fig. 2A). The PCA indicated a high correlation among antimicrobial traits (MIC) and oxygenated compounds, like OM and OS as previously reported for *Citrus* EOs (Randazzo, Jiménez-Belenguer, et al., 2016; Settanni et al., 2014).

3.4. Evaluation of the antibacterial activity of the EO-based sanitizer

According to the antibacterial results, EO6 was chosen to be prepared into a SAN solution to be evaluated as food contact surface sanitizer according to official methods. The SAN's efficacy was tested according to AOAC 960.09 and is reported in Table 3. In this case, the SAN containing 1% of EO6 was highly effective, inhibiting aproximately 8 log CFU/ml of *E. coli* and *S. enterica* after 30 and 60 seconds of contact time, respectively. According to method validation, a 99.999% (5 log CFU/ml) reduction was achieved for both strains within 30 seconds. Consequently, the developed SAN passed the validation recommended by the AOAC method.

Studies evaluating EOs for bacterial inhibition within food service environments remain somewhat limited (Phillips, 2016; Simões et al., 2010). Therefore, this SAN was further evaluated at various concentrations, cleanness conditions, contact times, and on different material surfaces commonly employed in food industries (Figure 3, Figure 4 and Table 6S).

As expected, the SAN's inhibitions were higher when tested at higher percentages (0.5<1<5 %) and for longer contact time (1<5<10 min) as reported by Messager, Hammer, Carson, and Riley (2005) for tea tree oil. The SAN was also tested on simulated clean and dirty surfaces (by preparing bacterial inocula in 0.3 and 3.0 g/l BSA, respectively, as in ISO 13697:2015). Figures 3 and 4 show titers of recovered *E. coli* and *S. enterica* on stainless steel, glass and polypropylene surfaces before, and after 1, 5, and 10 min treatment with a 5% SAN solution.

Titers of control samples were 5.75 ± 0.14 and 5.63 ± 0.25 log CFU/ml for *E. coli* and *S. enterica*, respectively. On clean stainless steel, the 5% SAN solution reduced *E. coli* counts by 1.38, 2.72, and 3.60 1og after 1, 5, and 10 min of exposure, respectively, while for *S. enterica* reductions of 0.32, 0.50 and 1.13 log were recorded. On clean glass, 0.77, 1.99 and 3.01 log reductions were

300 recorded for *E. coli* treated with the 5% SAN solution after 1, 5, and 10 min, respectively, and *S.*
301 *enterica* was reduced by 0.33, 0.43, and 1.13 log. On clean polypropylene, 5% SAN reduced 0.94,
302 2.59 and 3.46 log *E. coli* and 0.23, 0.43 and 1.03 log *S. enterica* after 1, 5, and 10 min, respectively.
303 Statistically significant inhibitions were reported for *S. enterica* after 10 min of contact with the 5%
304 SAN solution under clean working conditions for the three material tested, with reductions of 1.03-
305 1.13 log CFU/ml. Higher reductions have been reported by other authors when extending the time
306 of contact. For instance, reductions of 3.71 to 7.41 log CFU/cm^2 were reported for *Salmonella* spp.
307 biofilms on polypropylene treated for 1 h with 312 µg/ml (0.03%) of carvacrol (Amaral et al.,
308 2015). Similarly, approximately 6 log CFU/cm^2 reductions were achieved for *Salmonella* spp
309 attached on stainless steel after 10 min contact with 0.03% carvacrol (Engel et al., 2017).
310 Generally, Gram-negative bacteria are more resistant than Gram-positive bacteria to EOs (Nazzaro,
311 Fratianni, De Martino, Coppola, & De Feo, 2013), and, among Gram-negative bacteria ,*E. coli*
312 usually reported as more sensitive than *Salmonella* spp. (Semeniuc, Pop, & Rotar, 2017). The
313 SAN's limited activity against *Salmonella* could be explained by the EO's effect on some outer
314 membrane proteins involved in the formation of an efflux system (e.g. TolC), that may be up-
315 regulated by the EO and constitute a final mechanism of resistance, as observed for thymol
316 (Baucheron, Mouline, Praud, Chaslus-Dancla, & Cloeckaert, 2005).
317 In general, the results showed more effectiveness on clean surfaces than on dirty ones, and
318 significant differences ($p < 0.05$) were recorded among the different surface materials (Table 2S).
319 Regarding the latter, the higher disinfectant efficacy of sanitizers on smooth (i.e. steel) rather than
320 rough (i.e. plastic) surfaces has been previously reported (Lin, Sheu, Hsu, & Tsai, 2010).
321 On all clean surfaces tested, the 5% SAN solution was able to reduce *E. coli* counts by more than 3
322 log CFU/ml compared to the control (99.9%). In dirty conditions, the 5% SAN solution achieved
323 lower reductions (2.65 log CFU/ml on plastic). The presence of organic matter also reduced the
324 effectiveness of chemical sanitizers, such as sodium hypochlorite (Kich et al., 2004; Souza &
325 Daniel, 2005) or sodium dichloroisocyanurate (NaDCC) (Williams, Denyer, Hosein, Hill, &

Maillard, 2009), because the higher amount of proteins in dirty conditions may protect bacteria cells from the disinfectant action, as previously reported (Hammer, Carson, and Riley (1999) and Messager et al. (2005)).

The 5% SAN solution was effective against both bacterial strains when applied for 10 min (Figures 3 and 4). These different inhibitions between the two bacteria could depend on the species and strain tested, since various authors have reported heterogeneous antibacterial effects depending on the bacterial species (S. Burt, 2004; Fisher & Phillips, 2008) and strain (Settanni et al., 2014). For all the experiments, the ethanol used as a control did not show any significant inhibitory effect.

T. capitata EO demonstrated antimicrobial properties to certain extent, therefore, SAN improvement should be evaluated for example by the addition of stabilizers (S. A. Burt, Vlielander, Haagsman, & Veldhuizen, 2005).

Conclusions

Considering the increasing resistance of bacteria to chemical compounds and sanitizers, searching for natural antibacterial products is becoming a priority.

This study demonstrated the antimicrobial activity of *T. capitata* EO, and, for the first time, its potential use as a natural sanitizing product.

The EO-based sanitizer was developed by applying official methods (AOAC 960.09 and ISO 13697:2015) and testing different concentrations (0.25, 0.5 and 2.5%), cleanness conditions (clean and dirt), contact times (1, 5 and 10 minutes), and on stainless steel, glass and polypropylene surfaces commonly employed in food industries. Finally, a natural sanitizer containing 2.5% of *T. capitata* EO was effective against *E. coli* (> 3 log redution in all three clean material tested), but had limited effect on *S. enteridis* when evaluated on different food contact surfaces, suggesting an interesting potential of its application in real conditions even further improvements are needed to widen its efficacy against a wider range of bacterial pathogens.

352 **Acknowledgments**

353 This work was supported by the Spanish Ministry of Economy and Competitiveness (MINECO)
354 (RYC-2012-09950) and the Spanish National Institute for Agriculture and Food Research and
355 Technology (INIA) co-financed by the European Social Fund (Project RTA2014-00024-C03). GS
356 was supported by the "Ramon y Cajal" Young Investigator.

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534 **Table 1.** Main chemical compounds (>10%) characterizing extracted EOs by GC and GC–MS analysis.

Compound[a,b]	Class compound	IK	EO1	EO2	EO3	EO4	EO5	EO6	EO7	EO8	EO9
Sabinene	MH	980		0.34	0.42	2.11	t		3.13	34.41	0.14
β-Pinene	MH	982	1.47	0.66	0.46	0.53	0.58	0.03	17.28	2.2	1.12
p-Cymene	MH	1027	0.73	0.50	3.40	0.10		1.69		0.17	28.34
Limonene	MH	1033		0.89		1.13		0.05	30.14	3.69	t
1.8-Cineole	OM	1037	0.07		11.74	4.72	1.34				6.99
Artemisia ketone	OM	1065	57.54		3.15						
Linalool	OM	1107		4.87		0.35	4.19	0.64	1.20	21.27	0.28
Epoxy-ocimene <(E)->	OM	1140		34.01							
Camphor	OM	1149		7.96	31.43		0.87				
Cryptone	OM	1192									14.12
α-Terpineol	OM	1195		t		3.09	12.60	0.02	0.34	0.77	1.01
Citronellol	OM	1237					20.40				
Pulegone	OM	1245				14.15					
Geraniol	OM	1250					12.30		0.65		
cis-Chrysanthenyl acetate	OM	1267		28.35							
Geranial	OM	1269		t			1.20		11.91	0.02	
Carvacrol	OM	1317						91.56			
α-Terpineol acetate	OM	1353				32.59					
β-Copaen-4-α-ol	OS	1580			10.11						
Carvone acetate	OM	1574				10.29					
Spathulenol	OS	1580	1.32								17.99
Intermedeol	OS	1667	10.54	0.07							
Monoterpene hydrocarbons. (MH)			5.98	4.56	11.17	6.41	2.05	3.25	59.11	64.47	34.66
Oxygenated monoterpenes. (OM)			66.03	80.16	66.16	87.44	75.1	93.31	34.51	28.82	38.61
Sesquiterpene hydrocarbons. (SH)			0.6	5.05	2.9	3.56		2.17	3.15	1.66	0.83
Oxygenated sesquiterpenes. (OS)			21.35	0.85	14.31	0.30	1.52	0.53		4.25	21.82

Esters. (EST)	0.04		0.38	0.83					
Others. (OTH)		2.07	0.38	0.66	17.25	0.05	0.42		
Total identified (%)	94.00	92.69	95.30	99.19	95.92	99.31	97.19	99.20	95.92

[a]Compounds listed in order of elution in the ZB-5 column.

[b]The complete list of identified compounds is in Table 6S.

t. traces (<0.02%); IK. Kovats retention index relative to C_8–C_{25} n-alkanes on the ZB-5 column.

539 **Table 2.** Inhibitory activity of EOs tested by paper disc diffusion assay (PDDA) and minimum
540 inhibitory concentration (MIC)

	Escherichia coli		***Salmonella enterica***	
	PDDA (cm)	**MIC** (%)	**PDDA** (cm)	**MIC** (%)
EO1	1.00±0.00	nd	1.00±0.00	nd
EO2	1.10±0.00	nd	1.10±0.00	nd
EO3	1.43±0.19	0.5	1.60±0.18	1
EO4	1.58±0.10	1	1.33±0.10	1
EO5	1.88±0.15	1	1.88±0.12	1
EO6	2.75±0.35	0.05	2.47±0.28	0.05
EO7	1.45±0.06	0.5	1.38±0.05	>1
EO8	1.75±0.10	0.5	2.23±0.26	0.5
EO9	1.83±0.13	0.5	1.50±0.00	0.5

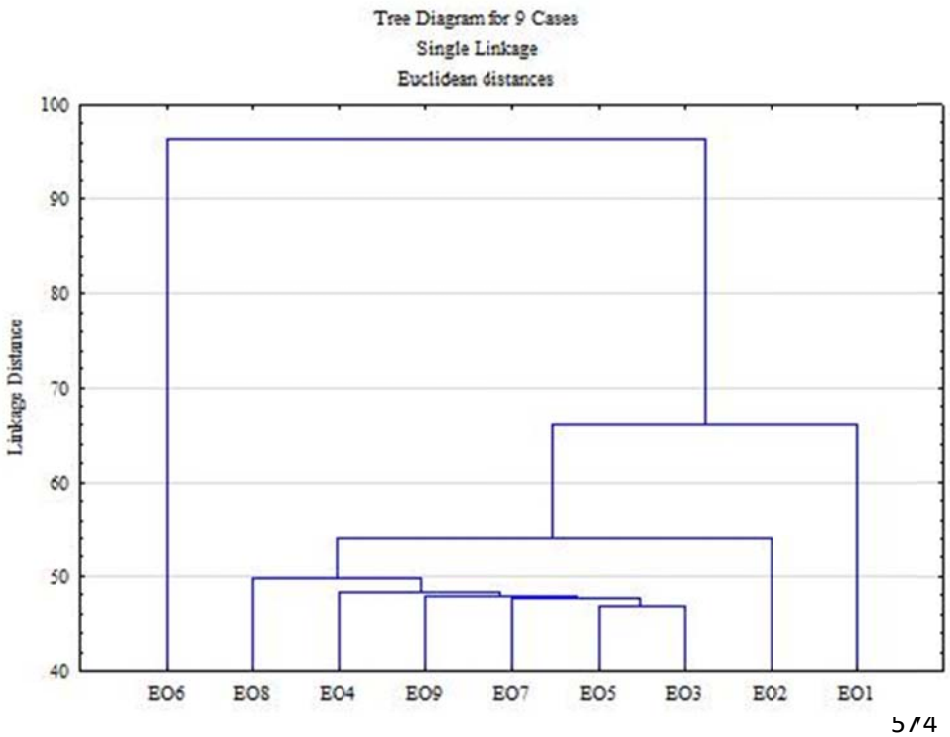
541
542 nd. not determined. The results are expressed in cm and represent the mean value of the inhibition haloes of four determinations (carried out in
543 duplicate and repeated twice) ± standard deviation.
544

545 **Table 3.** Evaluation of 1% natural sanitizing solution (SAN) against *Escherichia coli* and
546 *Salmonella enterica* after 30 and 60 seconds of contact time according to AOAC 960.09 standard
547 method.

	Escherichia coli				***Salmonella enterica***			
	30” Plate counts (log CFU/ml)	30” Reduction	60” Plate counts (log CFU/ml)	60” Reduction	30” Plate counts (log CFU/ml)	30” Reduction	60” Plate counts (log CFU/ml)	60” Reduction
Untreated	8.28±0.56	-	8.28±0.56	-	7.83±0.16	-	7.83±0.16	-
Ethanol 1%	8.00±0.13	0.28	8.05±0.25	0.23	7.72±0.03	0.11	7.65±0.03	0.18
SAN 1%	0	8.28	0	8.28	2.32±0.01	5.51	0	7.83

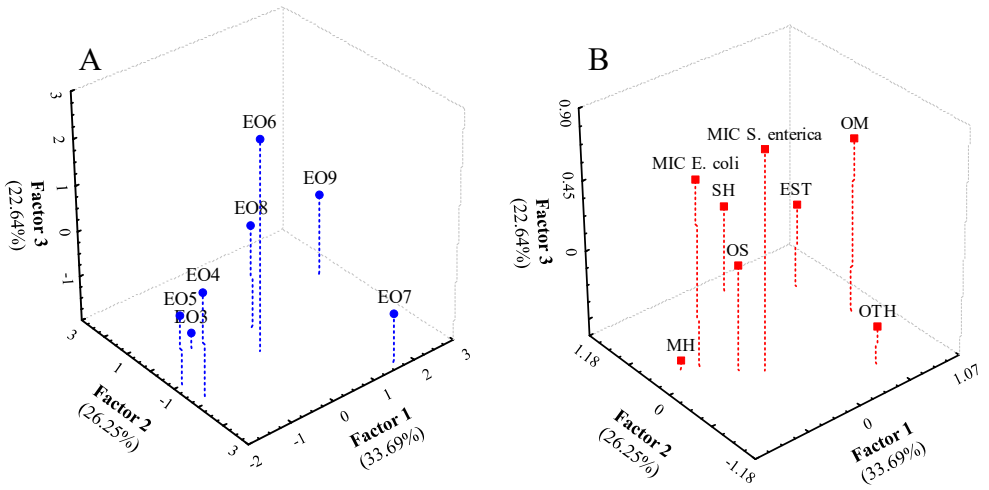
548
549

Figure 1. Hierarchical clustering analysis of the nine essential oils according to their chemical and antimicrobial characterizations.



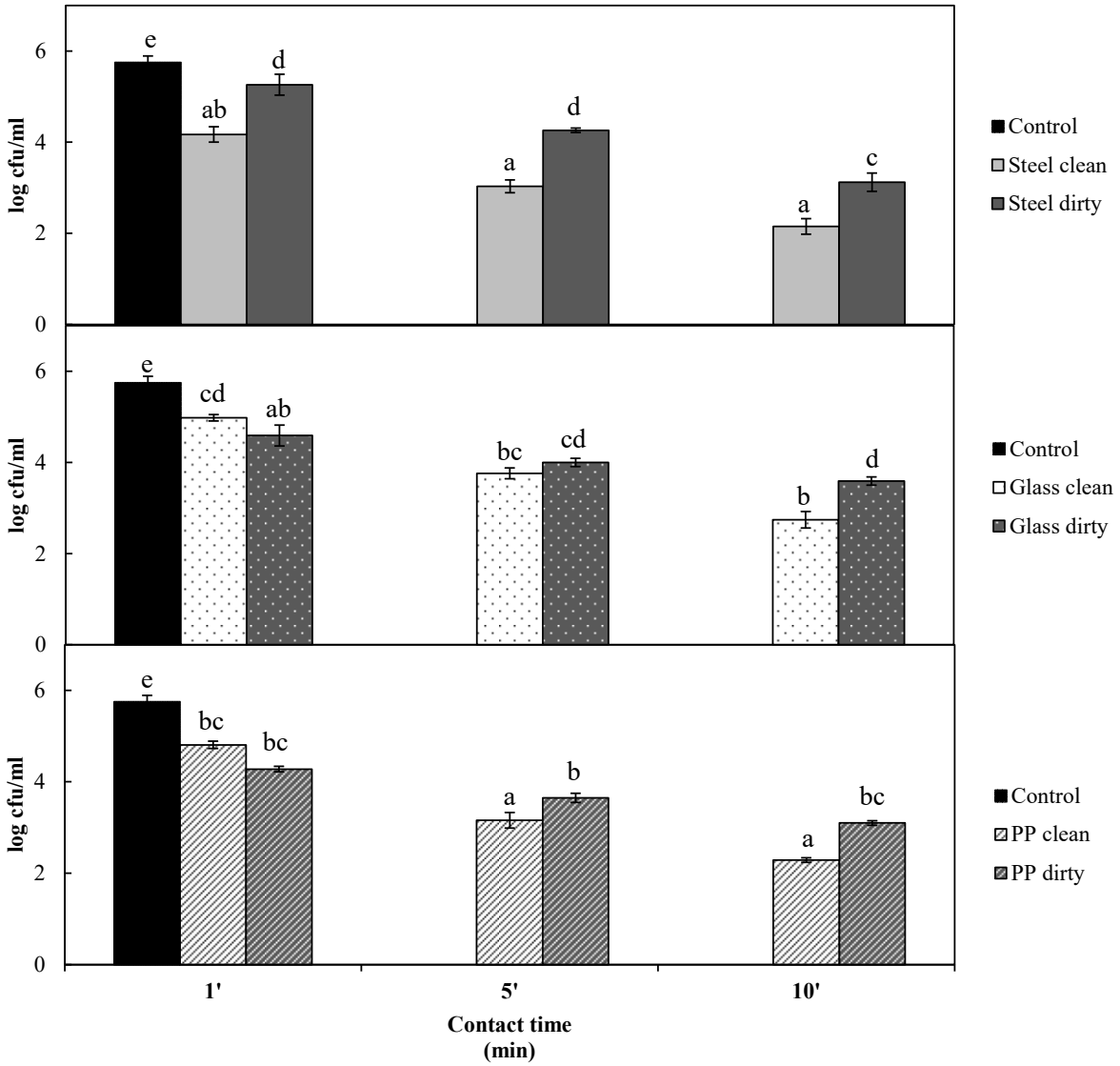
Abbreviations: EO1. *Eriocephalus africanus*; EO2. *Artemisia absinthium*; EO3. *Santolina chamaecyparissus*; EO4. *Mentha longifolia*; EO5. *Pelargonium odoratissimum*; EO6. *Thymbra capitata*; EO7. *Citrus limon*; EO8. *Citrus reticulata*; EO9. *Eucalyptus camaldulensis*.

Figure 2. Principal component analysis based on chemical compositions and antimicrobial activity of essential oils. Scatterplots show relationship between Factors and essential oils samples (score plot. A). and variables (loading plot. B).



Abbreviations: EO1. *Eriocephalus africanus*; EO2. *Artemisia absinthium*; EO3. *Santolina chamaecyparissus*; EO4. *Mentha longifolia*; EO5. *Pelargonium odoratissimum*; EO6. *Thymbra capitata*; EO7. *Citrus limon*; EO8. *Citrus reticulata*; EO9. *Eucalyptus camaldulensis*. MIC E. coli and S. enterica, minimum inhibitory concentration for *E. coli* and *S. enterica*. respectively; MH. monoterpene hydrocarbons; OM. oxygenated monoterpenes; SH. sesquiterpene hydrocarbons; OS. oxygenated sesquiterpenes; EST. esters; OTH. others.

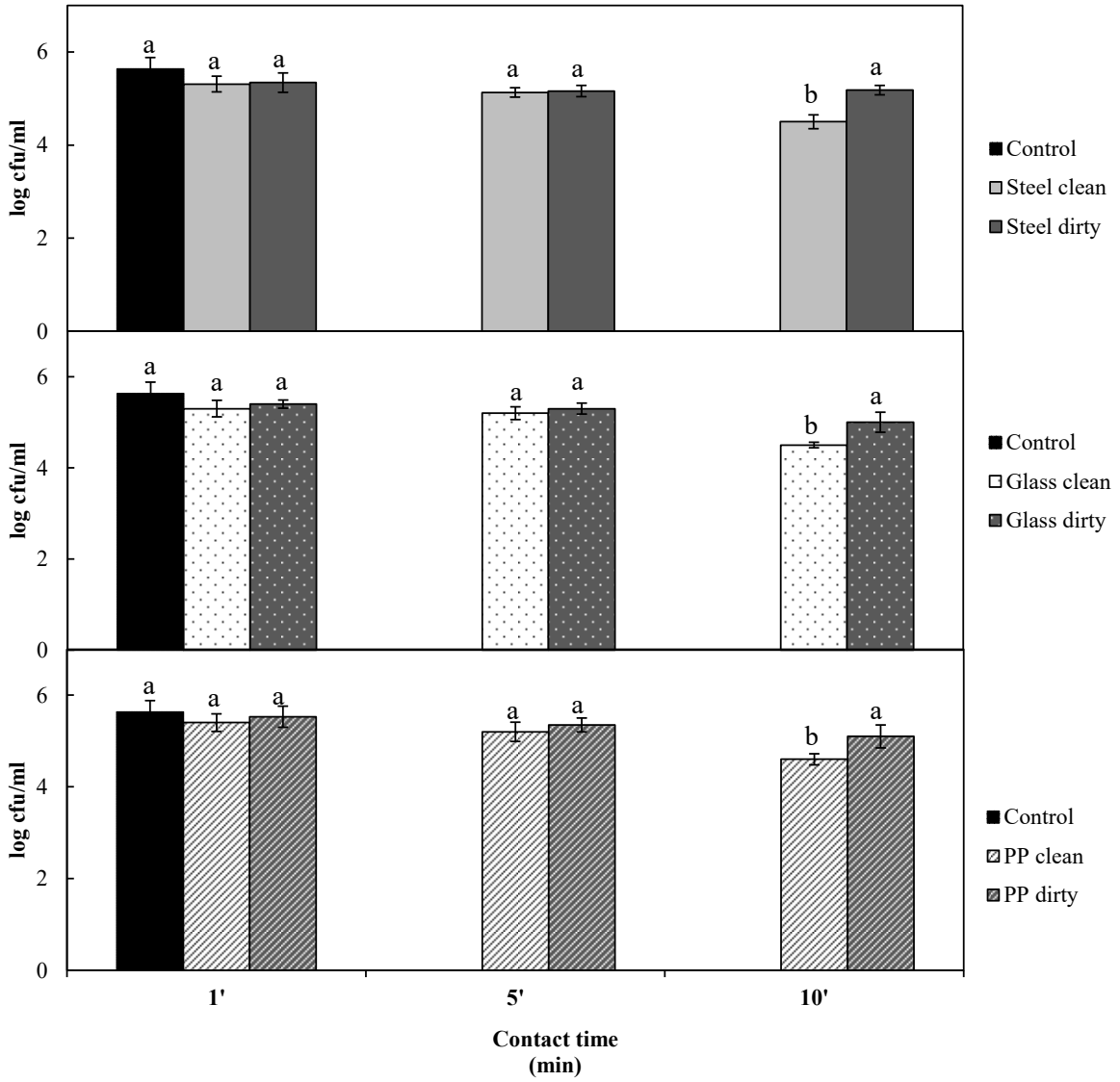
583 **Figure 3.** Bactericidal activity of 5% of a natural sanitizing solution (SAN) against *Escherichia coli*
584 on different food contact surfaces (stainless steel, glass and polypropylene, PP, discs) cleanness
585 conditions and contact times according to EN 13697:2015.



586
587 Error bars indicate standard errors of the means. For each contact time. samples with different
588 letters are statistically different according to the analysis of variance followed by Tukey's test
589 ($p \leq 0.05$).

590

Figure 4. Bactericidal activity of 5% of a natural sanitizing solution (SAN) against *Salmonella enterica* on different food contact surfaces (stainless steel, glass and polypropylene discs) cleanness conditions and contact times. according to EN 13697:2015.



Error bars indicate standard errors of the means. For each contact time. samples with different letters are statistically different according to the analysis of variance followed by Tukey's test ($p \leq 0.05$).